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# Targeting the A3 Adenosine Receptor for Cancer Therapy: Inhibition of Prostate Carcinoma Cell Growth by A3AR Agonist

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**Abstract.** *Background: Agonists to A3 adenosine receptor (A3AR) were shown to inhibit the growth of various tumor cell types. The present study demonstrates that a synthetic A3AR agonist, 1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purine-9-yl]-N-methyl-β-D-ribofura-nuronamide (IB-MECA), inhibits the growth of androgen-independent PC-3 prostate human carcinoma cells and illustrates the molecular mechanism involved. Materials and Methods: PC-3 prostate carcinoma cells were used. Cell growth was examined in vitro by the thymidine incorporation assay and in vivo by inoculating the tumor cells subcutaneously into nude mice and monitoring tumor size. The protein expression level in cells and tumor extracts was tested by Western blot analysis. Results: A decrease in the protein expression level of A3AR and the downstream effector PKAc was observed. Consequently, the GSK-3β protein level increased, resulting in the destabilization of β-catenin and the subsequent suppression of cyclin D1 and c-myc expression. IB-MECA treatment also induced down-modulation of the expression of NF-kB/p65, known to regulate the transcription of cyclin D1 and c-Myc. This chain of events occurred both in vitro and in vivo and suggests the use of the above-mentioned signaling proteins as markers to predict tumor cell response to A3AR activation. Conclusion: Taken together, we demonstrated that A3AR activation de-regulates the Wnt and the NF-kB signaling pathways resulting in the inhibition of prostate carcinoma cell growth.*

Activation of the Gi-protein-coupled A3AR has been involved in the inhibition of tumor cell growth (1-3). A3AR is highly expressed in tumor cells whereas low expression has been noted in a variety of normal cells (4-6). We recently examined the relationship between receptor fate upon activation and receptor functionality in melanoma cells. A3AR activation, with the synthetic agonist IB-MECA, induced rapid receptor internalization to the cytosol. The receptor was then degraded, subsequently re-synthesized and recycled to the cell surface to serve again as a functional receptor. These events generated the modulation of key proteins involved in the Wnt and the NF-kB signal transduction pathways. A decrease in cAMP production and expression of the downstream effector protein kinase A (PKA) and protein kinase B (PKB/Akt) was observed (7-9). We found that when PKA and PKB/Akt were inhibited, GSK-3β level was up-regulated. This led to the phosphorylation and ubiquitination of β-catenin and a decrease in the expression level of cyclin D1 and c-myc, resulting in melanoma cell growth inhibition (3). Moreover, a decline in the expression level of NF-kB was also noted consequent to PKB/Akt down regulation (9). These results were confirmed in an experimental murine model in which IB-MECA inhibited the growth of B16-F10 melanoma metastatic foci in the lung and the development of subcutaneous primary tumor (9). Interestingly, in tumor lesions derived from IB-MECA treated mice, A3AR expression and the level of key signaling proteins (GSK-3β, β-catenin, NF-kB, cyclin-D1 and c-Myc) were modulated in a pattern corresponding to that observed *in vitro*. These studies demonstrated that there is a direct correlation between A3AR activation, modulation of the signaling proteins and the inhibition of tumor cell growth. We therefore defined 5 of these proteins (PKA, GSK-3β, NF-kB cyclin-D1 and c-Myc) as protein markers to predict the response of tumor cells to A3AR activation both *in vitro* and *in vivo* (9).

Prostate cancer is a common disease in Western countries (10,11) and it is highly resistant to chemotherapy. There is still no effective cure for patients with advanced prostate cancer especially in cases of hormone-independent tumors (12). The molecular mechanisms involved in the initiation, progression and development of prostate cancer are largely unknown. Recently, the Wnt and the NF-kB signaling pathways have also

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been implicated in the development of prostate carcinoma (13,14). It thus led us to study the effect of IB-MECA on the growth of the human androgen-independent PC-3 prostatic carcinoma cell line and to follow-up the modulation of the 5 protein markers defined above, both *in vitro* and *in vivo*

## Materials and Methods

*Reagents.* IB-MECA and MRS 1523 were purchased from RBI/Sigma (Natick, MA, USA). For both reagents, a stock solution of 10mM was prepared in DMSO and further dilutions in RPMI medium were performed. RPMI, fetal bovine serum (FBS) and antibiotics for cell cultures were obtained from Beit Haemek, Haifa, Israel. Rabbit polyclonal antibodies against murine and human PKAc, c-myc and GSK-3β were purchased from Santa Cruz Biotechnology Inc., Ca, USA. The human and murine rabbit polyclonal antibodies against murine and human cyclin D1 and Rel-65 NF-κB were purchased from Chemicon, Ca, USA. Rabbit polyclonal antibodies against murine and human A3AR were purchased from Alph Diagnostics, San Antonio, USA.

*Tumor cells and proliferation assay.* PC-3 cells derived from a human androgen-independent prostate cancer cell line (American Type Culture Collection, Manassas, Virginia, USA) were grown in RPMI 1640 penicillin, streptomycin, 2 mM. L-glutamine and 10% fetal bovine serum (FBS). The cells were maintained in T-75 flasks at 37°C in a 5% $CO_2$ incubator and transferred to a freshly prepared medium twice weekly. For all studies serum-starved cells were used. FBS was omitted from the cultures for 18 hours and the experiment was carried out on monolayers of cells in RPMI medium supplemented with 1% FBS in a 37°C, 5% $CO_2$ incubator.

[$^3$H]-thymidine incorporation assay was used to evaluate cell growth. PC-3 cells ($1.5x10^4$/ml) were incubated with IB-MECA (0.01μM-10μM) in 96-well microtiter plates for 24 hours. To test whether IB-MECA exerted its effect on tumor cells through binding to A3AR, an antagonist to A3AR, MRS-1523 (0.1μM), was added to the cell cultures in the presence of IB-MECA. Cultures of PC-3 cells that were incubated in the presence of MRS-1523 only served as controls. For the last 18 hours of incubation, each well was pulsed with 1μCi [$^3$H]-thymidine. The cells were harvested and the [$^3$H]-thymidine uptake was determined in an LKB liquid scintillation counter (LKB, Piscataway, NJ, USA). These experiments were repeated at least 10 times.

*Western blot analysis.* To detect the level of expression of A3AR, PKA, GSK-3β, β-catenin, c-myc and cyclin D1, protein extract from IB-MECA treated or untreated serum-starved PC-3 cells were utilized. The cells were incubated in the presence and absence of IB-MECA for 15 minutes at 37°C. At the end of the incubation period, the cells were then rinsed with ice-cold PBS and transferred to ice-cold lysis buffer (TNN buffer, 50mM Tris buffer pH=7.5, 150mM NaCl, NP 40 0.5% for 20 minutes). Cell debris were removed by centrifugation for 10 minutes, at 7500xg. The supernatant was utilized for Western blot analysis. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. Equal amounts of the sample (50μg) were separated by SDS-PAGE, using 12% polyacrylamide gels. The resolved proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked with 1% bovine serum albumin and incubated with the desired primary antibody (dilution 1:1000) for 24 hours at 4°C. The blots were then washed and incubated with a secondary antibody for 1hour at room temperature. Bands were recorded using BCIP/NBT color development kit (Promega, Madison,W1, USA). The densitometry of protein expression was normalized against β-actin and expressed as % of control (0-time).

*In vivo studies.* The mice were maintained on a standardized pelleted diet and supplied with tap water. Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Can-Fite BioPharma, Petah Tikva, Israel.

Nude male Balb/C mice, aged 2 months, weighing an average of 25g were obtained from Harlan Laboratories, Jerusalem, Israel. PC-3 prostate carcinoma cells ($2.5x10^6$) were subcutaneously injected into the flank of the mice. When the tumor reached 150-200mm$^3$ in size, the animals were randomly assigned into different experimental groups. Two types of experiments were set up:

A. a study in which the effect of IB-MECA on tumor growth was evaluated in mice in which the tumor reached a size of 150-200mm$^3$. Treatment was given orally once daily for 26 days. This experiment included two groups:

1. Vehicle
2. IB-MECA (10μg/kg body weight).

Tumor size (width (W) and length (L)) was measured twice weekly with a caliber and calculated according to the following formula: Tumor Size = $(W)^2xL/2$. Each group contained 10 mice.

B. a study in which the effect of IB-MECA on the expression of tumor markers was evaluated shortly after one treatment in tumor-bearing mice. This experiment included three groups:

1. Vehicle-control.
2. IB-MECA (10μg/kg body weight) given once. Mice were sacrificed after 2hours.
3. IB-MECA (10μg/kg body weight) given once. Mice were sacrificed after 24hours.

At the end of each experiment the mice were sacrificed and tumors were excised, protein extracts were prepared as described above and analyzed for the expression profile of A3AR and the marker proteins (PKA, NF-kB,GSK-3β, β-catenin and cyclin D1).

*Statistical analysis.* The results were evaluated using the Student's *t*-test, with statistical significance at $p<0.05$. Comparison between the mean value of different experiments was carried out.

## Results

*IB-MECA inhibits PC-3 growth in vitro and in vivo.* To evaluate the direct anti-proliferative effect of IB-MECA on the human androgen-independent PC-3 prostatic carcinoma cell line *in vitro*, we used the thymidine incorporation assay. IB-MECA exerted a dose-dependent inhibitory effect on the prostate carcinoma cells. The inhibition of cell growth was statistically significant at all concentrations tested ($p<0.001$). The A3AR antagonist MRS1523 reversed the inhibitory effect of IB-MECA, demonstrating that tumor growth suppression was specifically mediated through A3AR (Figure 1a).

*In vivo*, the treatment with IB-MECA started when the subcutaneously transplanted PC-3 tumors had grown to a volume of 150-200 mm$^3$. As shown in Figure 2a and b, IB-MECA sup-

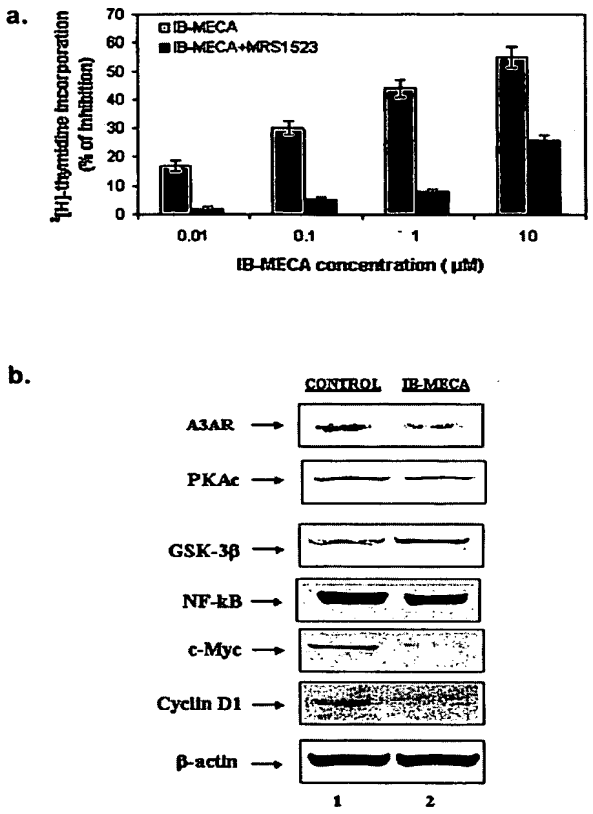



Figure 1. *(a) IB-MECA induces a dose-dependent inhibitory effect on the proliferation of PC-3 prostate carcinoma cells. PC-3 prostate carcinoma cells were depleted from serum for 18hours and treated with vehicle (control) or with various IB-MECA concentrations (0.01μM-10μM) in the presence of 1% FBS for 24hours. Cell proliferation was measured by [$^3$H]-thymidine incorporation assay. A3AR antagonist MRS-1523 (0.1μM) neutralized the inhibitory effect of IB-MECA. The data points are mean±SEM values from four independent experiments. (b) Expression level of A3AR and protein markers in PC-3 cells upon exposure to IB-MECA. Immunoblots showing the effect of 0.01μM IB-MECA on the expression level of A3AR, PKAc, GSK-3μ NF-kB, c-Myc and cyclin D1 in PC-3 prostate carcinoma cells. Serum starved cells (for 18 hours) were treated for 15 minutes with IB-MECA in the presence of 1% FBS.*

pressed growth of PC-3 tumors during the 26 days of treatment. At the end of the experiment, the mean volume of PC-3 tumors treated with IB-MECA was 69±37 mm$^3$, being significantly smaller than that in control group which measured 340±59 mm$^3$, the inhibition of tumor growth corresponding to 79.7% ($p<0.0001$, Figure 2a).

*IB-MECA modulates tumor marker proteins upon A3AR activation.* Shortly after A3AR activation with IB-MECA *in vitro*, the expression level of the receptor protein was down-regulated. Additional marker proteins, downstream to A3AR activation, were modulated, *i.e.*, PKA, NF-kB, c-Myc and cyclin D1 expression levels were decreased whereas GSK-3β level was up-regulated (Figure 1b).

In tumor lesions excised from mice treated daily for 26 days with IB-MECA, Western blot analysis revealed down-regulation of A3AR, PKAc, cyclinD1 and c-myc and up-regulation of GSK-3β expression level (Figure 2c). The level of the housekeeping protein β-actin did not change.

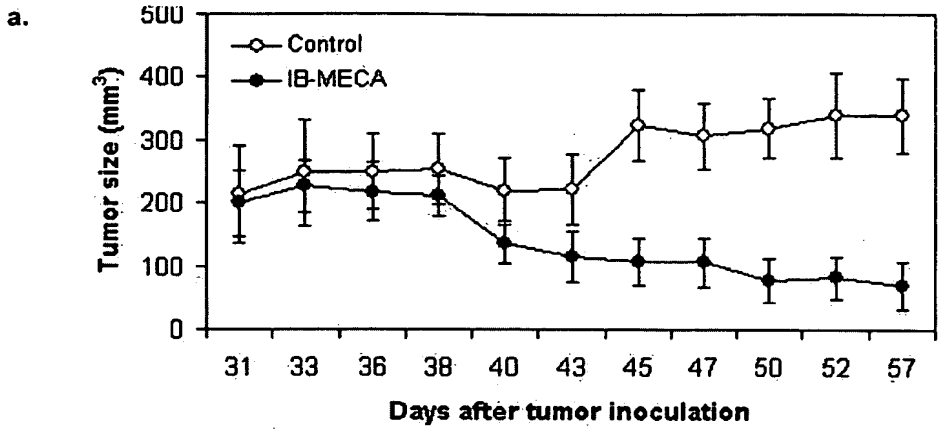
To explore the response of the above mentioned tumor proteins to one treatment of IB-MECA, mice with an already established tumor were treated only once with IB-MECA. Two hours after treatment, a marked down-regulation of A3AR, PKA, β-catenin, NF-kB, c-Myc and cyclin D1 was noted. Interestingly, 24 hours after IB-MECA administration, A3AR protein expression level was fully recovered to the control level, whereas the expression level of the other proteins was only partially recovered, and was lower than the control group.

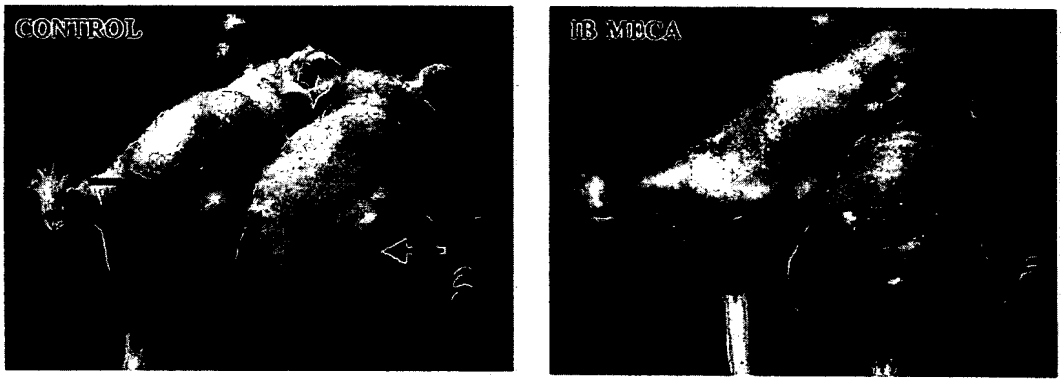
## Discussion

The present study describes the ability of IB-MECA, a synthetic A3AR agonist, to inhibit the growth of prostate carcinoma cells in vitro and *in vivo*. A3AR belongs to the family of the Gi-protein-associated cell surface receptors. Receptor activation leads to internalization and the subsequent inhibition of adenylyl cyclase activity, cAMP formation and protein kinase Ac (PKAc) expression (15, 16). IB-MECA is a potent, stable and specific A3AR agonist due to a substitution at the N6 and 5' positions of adenosine. This structure protects the molecule against rapid metabolization by adenosine deaminase and further enhances its affinity to A3AR (17). A3AR expression level was found to be low in most body tissue, whereas tumor cells such as melanoma, T cell lymphoma and pineal tumor cells, significantly express A3AR (4-6). Receptor exhibition and spread is not the only factor determining cell response to a specific ligand. An additional parameter is the exhibition of A2A and A2B adenosine cell surface receptors, known to elicit opposite effects to that of A3AR. At high concentrations, A3AR agonists may also activate A2A and A2B adenosine receptors, affecting the balance of the response (18, 19).

In the present study, the dose-dependent growth inhibition observed in the PC-3 cells *in vitro* was obtained at low concentrations and was counteracted by the antagonist MRS1523. *In vivo*, IB-MECA generated the suppressive effect on tumor growth also at a low-dose (10μg/kg body weight). It is assumed that since IB-MECA possesses high affinity to A3AR (0.4nM), it activates this receptor exclusively at low concentrations.

Shortly upon IB-MECA activation, down-regulation of A3AR protein expression level was noted *in vitro*. This observation was confirmed in the *in vivo* studies in which we treat-



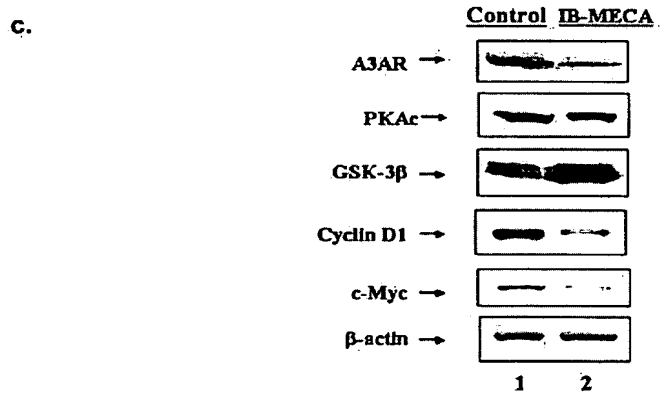




Figure 2. *(a) Inhibition of prostate carcinoma cell growth in mice and modulation of tumor protein markers in tumor lesions. (a) PC-3 prostate carcinoma cells ($2.5x10^6$) were subcutaneously injected into the flank of nude mice. One group was treated with IB-MECA (10μg/kg body weight) daily orally, starting when the tumor reached a size of 150-200 $mm^3$ and the other, treated with vehicle only, served as control. (b) Representative mice from the control (left) and the IB-MECA (right) treated mice, showing the difference in tumor size in the two groups. (c) Immunoblots showing the effect of IB-MECA on the level of A3AR, PKAc, GSK-3β, cyclin D1 and c-Myc in protein extracts derived from tumor lesions of prostate carcinoma bearing mice (description of the experiment is detailed in a).*

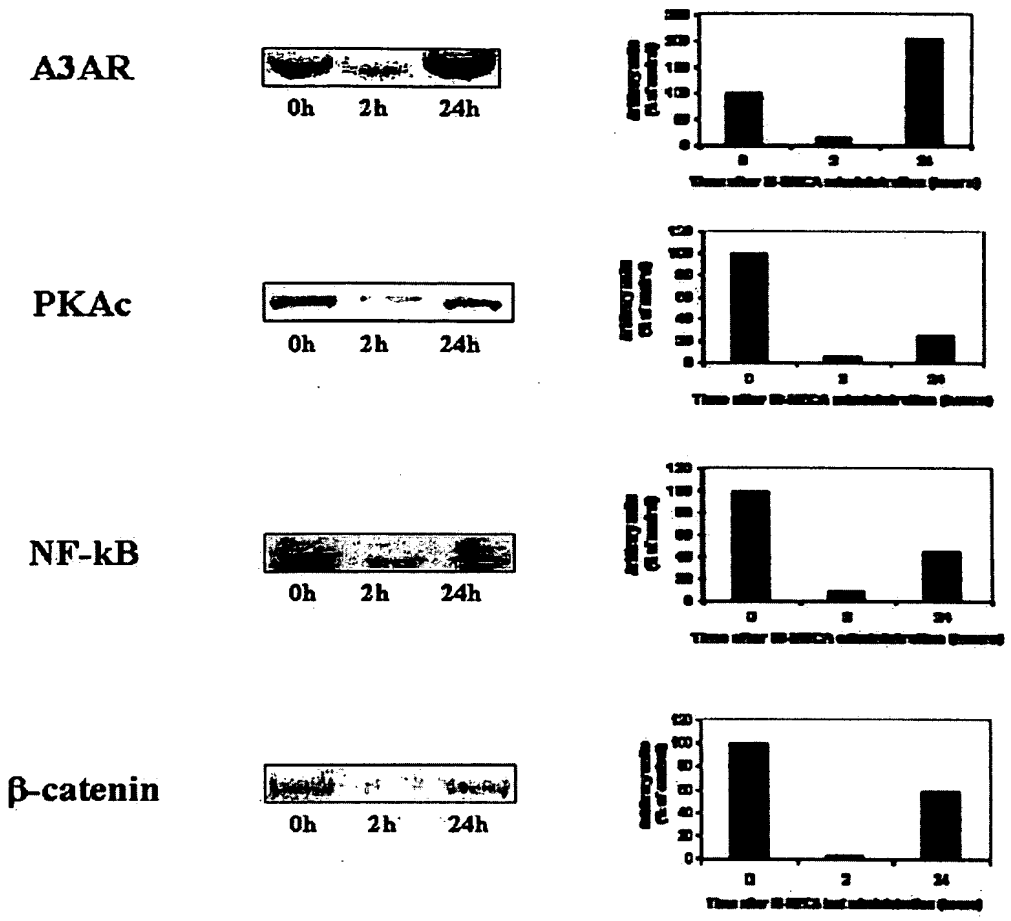



Figure 3. *Modulation of tumor protein markers in tumor lesions derived from IB-MECA-treated mice. The effect of IB-MECA (one treatment only, for 2 hours and 24 hours) on the expression of tumor protein markers was evaluated in tumor lesions excised from prostate carcinoma-bearing mice. Immunoblots showing the effect of IB-MECA on A3AR, PKAc, NF-kB, β-catenin, cyclin D1 are presented.*

ed tumor-bearing mice with IB-MECA. Receptor down-regulation is a general mechanism typical of Gi protein receptors. This family of receptors responds to ligand activation by receptor internalization (to the cytosol), degradation, re-synthesis and recycling to the cell surface (20). During these events, receptor desensitization/re-sensitization takes place and different signaling pathways are initiated (21, 22). We may suggest that the down-regulation of receptor expression in this study represents the rapid response of the prostate cells to agonist stimulation and the initiation of downstream responses.

Indeed, the prostate cells responded to A3AR activation by a decrease in PKAc level both *in vitro* and *in vivo*. PKAc is an effector protein involved in the initiation/regulation and cross talk between various signaling pathways. It phosphorylates and inactivates the enzyme GSK-3β (23), a key element in the Wnt signaling pathway (24). GSK-3β suppresses mammalian cell proliferation and survival by phosphorylating the cytoplasmic protein β-catenin, leading to its ubiquitination. GSK-3β in its inactive form does not phosphorylate β-catenin. The latter accumulates in the cytoplasm and subsequently translocates to the nucleus where it associates with Lef/Tcf to induce cyclin-D1 and c-myc transcription (25). In the present study we found that up-regulation of GSK-3β correlated with down-regulation of β-catenin, cyclin D1 and c-Myc. Davies *et al.* reported that there were no mutations within the binding regions between β-catenin and GSK-3β in PC-3 prostate carcinoma cells (26). Therefore, we concluded that there is an involvement of the Wnt pathway in the response of these cells to A3AR activation.

The expression level of NF-kB was down-regulated in both *in vitro* and *in vivo* studies. NF-kB is also linked to the effector protein PKAc. The most abundant form of NF-kB is a het-

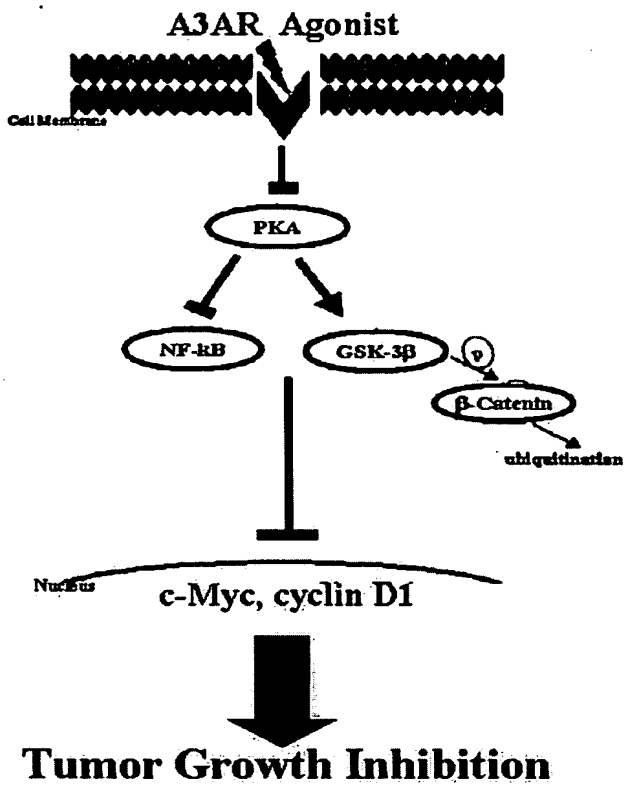



Figure 4. *Schematic representation of signaling pathways that mediate A3AR inhibition of melanoma cell growth.*

erodimer of p50 and p65 (Rel A) subunits in which the p65 contains the transcription activation domain. PKAc regulates the transcriptional activity of NF-kB by phosphorylating the p65 subunit of NF-kB, enabling its association with the co-activator CBP/p300 and the efficient transcriptional activity (27).

Previous reports have suggested that PC-3 prostate carcinoma cells and the androgen receptor-negative cell line (DU-145) have constitutive NF-kB activity (27, 28). Thus, the IB-MECA's capability to suppress NF-kB expression may serve as part of the mechanism through which it exerts an inhibitory effect on androgen-independent cells.

*In vivo*, the protein markers were significantly modulated upon a single or chronic exposure of the tumors to IB-MECA. One conclusion that can be drawn from this phenomenon is that these protein markers may serve as biomarkers for predicting the response of the tumor to IB-MECA in the host. These results provide a rationale to examine the protein markers in patients on IB-MECA treatment.

Collectively, these results suggest that IB-MECA inhibits the growth of prostate cancer cells *via* modulation of key proteins involved in the Wnt and NF-kB signaling pathway. These results corroborate our findings in other types of neoplasias (melanoma and colon carcinoma) and propose the use of A3AR agonists for the management of human prostate cancer.

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**Hepatoma**

Male Sprague-Dawley rats (Harlan Laboratories, Jerusalem, Israel) weighing an average of 200-250 gr were used. Subxyphoid laparatomy, 1.5-2 cm in length was performed to expose the left and right lobes of the liver. Using a 27G needle, a suspension containing $5x10^6$ of N1-S1 hepatocellular carcinoma cells in 100μL PBS were injected into one of the hepatic lobes under the liver capsule. IB-MECA at a dose of 100 μg/kg body weight was administered orally once daily, starting 24 hours after tumor cells' inoculation. The control group was treated daily, orally, with the vehicle only. Rats were sacrificed after 15 days, tumor lesions were excise and protein was extracted for measurements cell growth regulatory proteins levels.

Figure 1A shows an example of tumor size after 15 days of daily treatment with IB-MECA of rats inoculated with N1S1 murine hepatocelular carcinoma cells Figure. 1B presents the modulation of cell growth regulatory proteins (PKB/Akt, IKK$\alpha/\beta$, NF-$\kappa$B and TNF-$\alpha$) derived from the tumorlesions. the tumor lesions agonist

Figure 1A

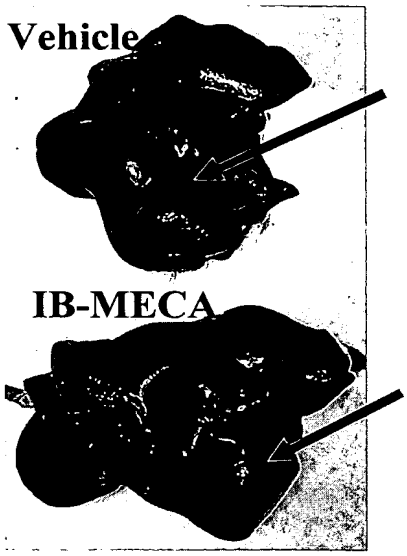
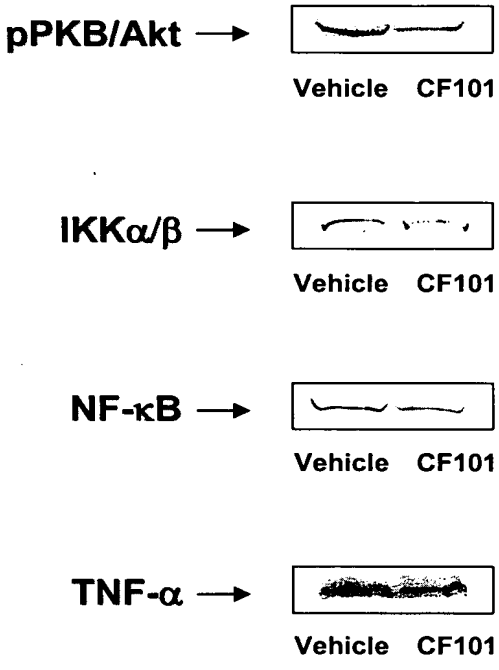



Figure 1B.



Multiple Sclerosis

Experimental Autoimmune Encephalomyelitis serves as a model for Multiple Sclerosis. Female Lewis rats (Harlan Laboratories, Jerusalem, Israel) 8 weeks old were used. An emulsion consisting of the following for each rat: 100 μg myelin basic protein (MBP) from guinea pig (M2295; Sigma), 0.1 ml Complete Freund's adjuvant (CFA; F5506, Sigma), and 0.2 mg of *Mycobacterium tuberculosis* H37 Ra (*M. tuberculosis*, 3114, Difco) was injected into the medial footpad of each hind limbs of the rats. IB-MECA at a dose of 10 μg/kg body weight was administered orally twice daily starting day 7 after disease induction. The rats developed clinical EAE symptoms which were graded into the following categories: 0, no neurological symptoms; 1, loss of tail tonus and paralysis of the whole tail; 2, hind limbs weakness; 3, hind limbs paralysis; 4, quadriplegia; 5, moribund.

Figure 1A shows the clinical score of the EAE. in the IB-MECA treated group vs. the vehicle treated group.

Figure. 1B presents the modulation of cell growth regulatory proteins ( total and phosphorylated GSK-3$\beta$, IL-10 and TNF-$\alpha$) derived from the spinal cord of EAE rats

Figure 1A

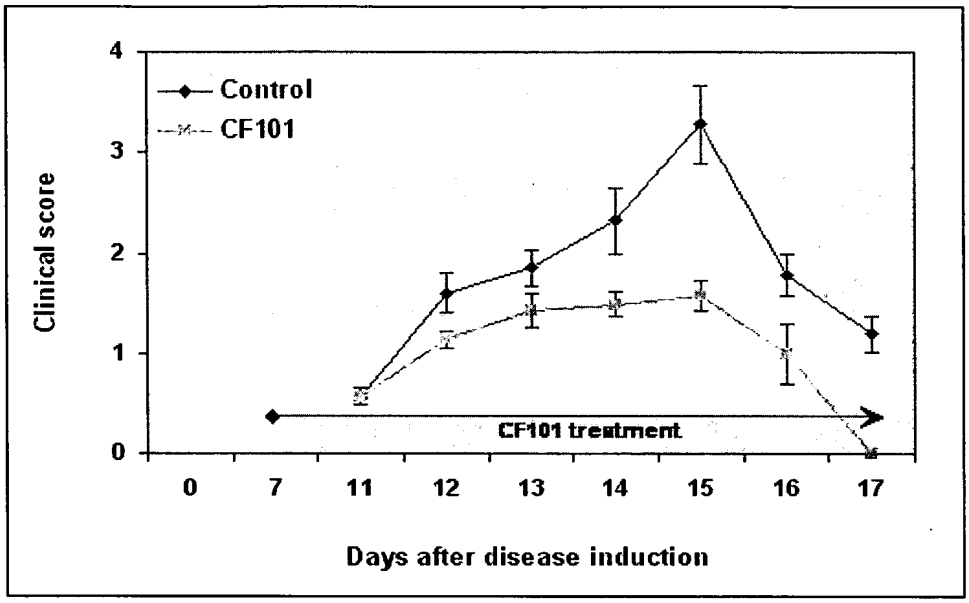
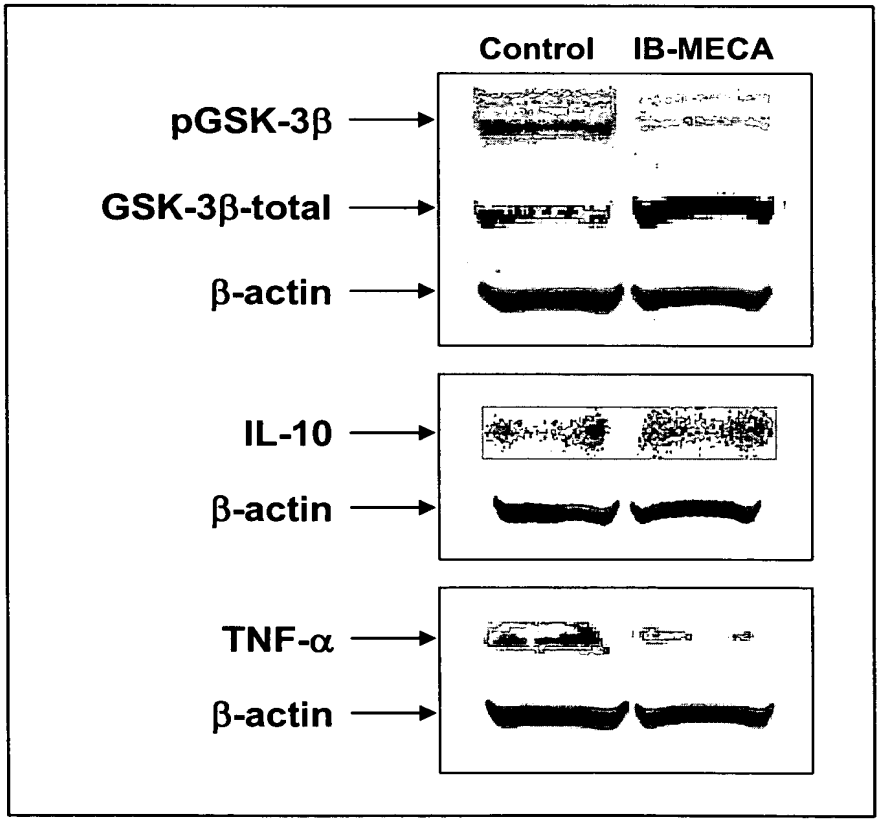



Figure 1B.



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# An agonist to the $A_3$ adenosine receptor inhibits colon carcinoma growth in mice via modulation of GSK-3$\beta$ and NF-$\kappa$B

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**$A_3$ adenosine receptor ($A_3$AR) activation with the specific agonist CF101 has been shown to inhibit the development of colon carcinoma growth in syngeneic and xenograft murine models. In the present study, we looked into the effect of CF101 on the molecular mechanisms involved in the inhibition of HCT-116 colon carcinoma in mice. In tumor lesions derived from CF101-treated mice, a decrease in the expression level of protein kinase A (PKA) and an increase in glycogen synthase kinase-3$\beta$ (GSK-3$\beta$) was observed. This gave rise to downregulation of $\beta$-catenin and its transcriptional gene products cyclin D1 and c-Myc. Further mechanistic studies *in vitro* revealed that these responses were counteracted by the selective $A_3$AR antagonist MRS 1523 and by the GSK-3$\beta$ inhibitors lithium and SB216763, confirming that the observed effects were $A_3$AR and GSK-3$\beta$ mediated. CF101 downregulated PKB/Akt expression level, resulting in a decrease in the level and DNA-binding capacity of NF-$\kappa$B, both *in vivo* and *in vitro*. Furthermore, the PKA and PKB/Akt inhibitors H89 and Worthmannin mimicked the effect of CF101, supporting their involvement in mediating the response to the agonist. This is the first demonstration that $A_3$AR activation induces colon carcinoma growth inhibition via the modulation of the key proteins GSK-3$\beta$ and NF-$\kappa$B.**
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**Keywords:** $A_3$ adenosine receptor; CF101; colon carcinoma; $\beta$-catenin; GSK-3$\beta$

## Introduction

The $A_3$AR is a $G_i$-protein-coupled receptor containing seven $\alpha$ helical spanning membrane domains. $A_3$AR was found to be expressed in different tumor cell lines, including Jurkat T, pineal gland, astrocytoma, melanoma as well as colon and prostate carcinoma (Gessi *et al.*, 2001; Merighi *et al.*, 2001; Suh *et al.*, 2001; Trincavelli *et al.*, 2002a; Fishman *et al.*, 2003; Madi *et al.*, 2003; Ohana *et al.*, 2003). $A_3$AR activation leads to inhibition of adenylyl cyclase activity, cAMP formation and PKA expression, resulting in the initiation of various signaling pathways which may include the MAPK and the PI3K (Poulsen and Quinn, 1998; Olah and Stiles, 2000; Trincavelli *et al.*, 2002b).

Our earlier studies demonstrated that melanoma cells highly express $A_3$AR, and suggested that it may serve as a target for tumor growth inhibition. $A_3$AR activation by the synthetic agonist 1-deoxy-1-[6-[[(3-iodophenyl)-methyl]amino]-9*H*-purine-9-yl]-*N*-methyl-$\beta$-D-ribofuranuronamide (IB-MECA) inhibited the growth of melanoma both *in vitro* and *in vivo* (Fishman *et al.*, 2001, 2002a, b, 2003; Ohana *et al.*, 2001; Madi *et al.*, 2003). The mechanistic pathway involved downregulation of the Wnt signaling pathway. It was found that IB-MECA inhibited the expression of PKAc and PKB/Akt, thereby preventing the phosphorylation and inactivation of GSK-3$\beta$. Consequently, GSK-3$\beta$ was shown to phosphorylate $\beta$-catenin and prevent its translocation to the nucleus, resulting in downregulation of cyclin D1 and c-Myc (Fishman *et al.*, 2002b; Madi *et al.*, 2003). PKB/Akt is also known to control NF-$\kappa$B level by phosphorylating downstream proteins, which in turn release NF-$\kappa$B from its complex (Madrid *et al.*, 2001). Similar to $\beta$-catenin, NF-$\kappa$B translocates to the nucleus, where, among other genes, it induces the transcription of c-Myc and cyclin D1 (Joyce *et al.*, 2001).

Our previous studies showed that CF101 is efficacious in suppressing the growth of primary and liver metastasis of CT-26 colon carcinoma cells in syngeneic experimental tumor models in mice (Ohana *et al.*, 2003). In addition, CF101 inhibited the growth of subcutaneous HCT-116 human colon carcinoma cells in a xenograft model in mice.

Aberrant activation of Wnt signaling, caused by mutations in $\beta$-catenin or APC, is a critical event in the development of colorectal tumors. In these cases, GSK-3$\beta$ fails to phosphorylate $\beta$-catenin, which accumulates in the cytoplasm. $\beta$-catenin then translocates to the nucleus where, in association with Lef/Tcf, it induces the transcription of cyclin D1 and c-Myc (Morin, 1999).

The present study is focused on the molecular mechanism involved in the inhibition of colon carcinoma growth by CF101. We explored the signaling

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modulation of GSK-3$\beta$ and NF-$\kappa$B, both of which are affected by PKB/Akt (which is downstream to PI3K) and are known to regulate the level of the important oncogenes cyclin D1 and c-Myc. A major role for GSK-3$\beta$ in mediating these responses is discussed.

## Results

### *CF101 inhibits colon carcinoma growth* in vivo *and modulates the expression level of $A_3AR$ and downstream cell growth-regulatory proteins in tumor lesions*

HCT-116 colon carcinoma cells were engrafted subcutaneously into nude mice. When tumor reached the size of 150–200 mm$^3$, the mice were treated daily orally with CF101. Tumor growth was suppressed in the CF101-treated group in comparison to the vehicle-treated group (Figure 1a). On the day of study termination, 52 + 6.1% ($P<0.001$) tumor growth inhibition was observed. To evaluate the effect of chronic CF101 treatment on $A_3AR$ expression and downstream cell growth-regulatory proteins, extracts were prepared from tumor lesions and subjected to Western blot (WB) analysis. In the group of mice killed 2 h after the last treatment, the expression level of $A_3AR$, PKAc, $\beta$-catenin, NF-$\kappa$B, c-Myc and cyclin-D1 was downregulated, whereas GSK-3$\beta$ was upregulated. In the group of mice killed 16 h after the last treatment, $A_3AR$ expression was similar to that of the vehicle-treated group. Interestingly, in this group, most of the cell growth-regulatory proteins were decreased in comparison to the control group, indicating that continuous downregulation is achieved upon chronic CF101 treatment. Taken together, these data show that receptor downregulation occurs shortly (2 h) after CF101 treatment, leading to modulation of downstream proteins, and that $A_3AR$ was not desensitized despite chronic activation (over a 20-day period). The expression of the receptor returned to normal levels 16 h after CF101 administration, demonstrating that, even after chronic activation, the receptor is fully expressed (Figure 1b).

### *CF101 modulates the expression level of $A_3AR$ and downstream cell growth-regulatory proteins* in vitro

To further study the association between $A_3AR$ activation and the expression of downstream cell growth-regulatory proteins, HCT-116 colon carcinoma cells were incubated in the presence of CF101 (10 nM) for 15 min. Proteins were extracted and analysed by WB. Similar effects of CF101 to those seen *in vivo* were recorded. The expression level of the two kinases PKAc and PKB/Akt was downregulated, while the expression of their downstream substrate GSK-3$\beta$ was upregulated. The levels of the coactivator $\beta$-catenin and the downstream target genes cyclin D1 and c-Myc were decreased (Figure 2a). To confirm that these responses are mediated via the $A_3AR$, the antagonist MRS 1523 was introduced to the culture system. The antagonist counteracted the effect of CF101, thereby retaining the control levels of PKAc, GSK-3$\beta$ and cyclin D1, demonstrating the specificity of the response (Figure 2b). To further elucidate the role of PKA and

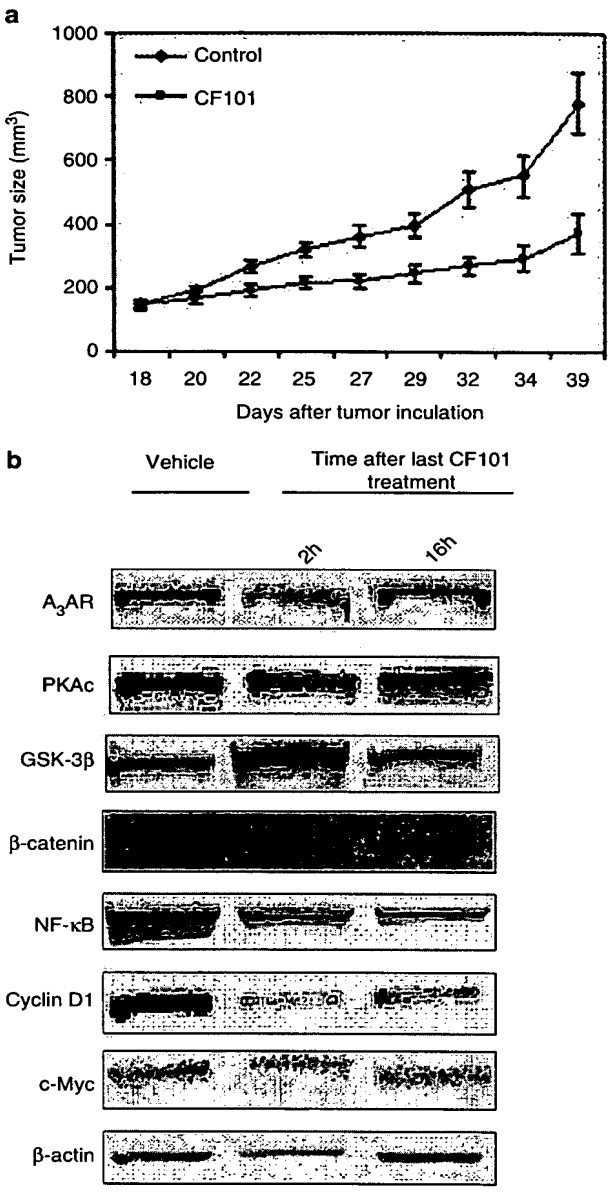
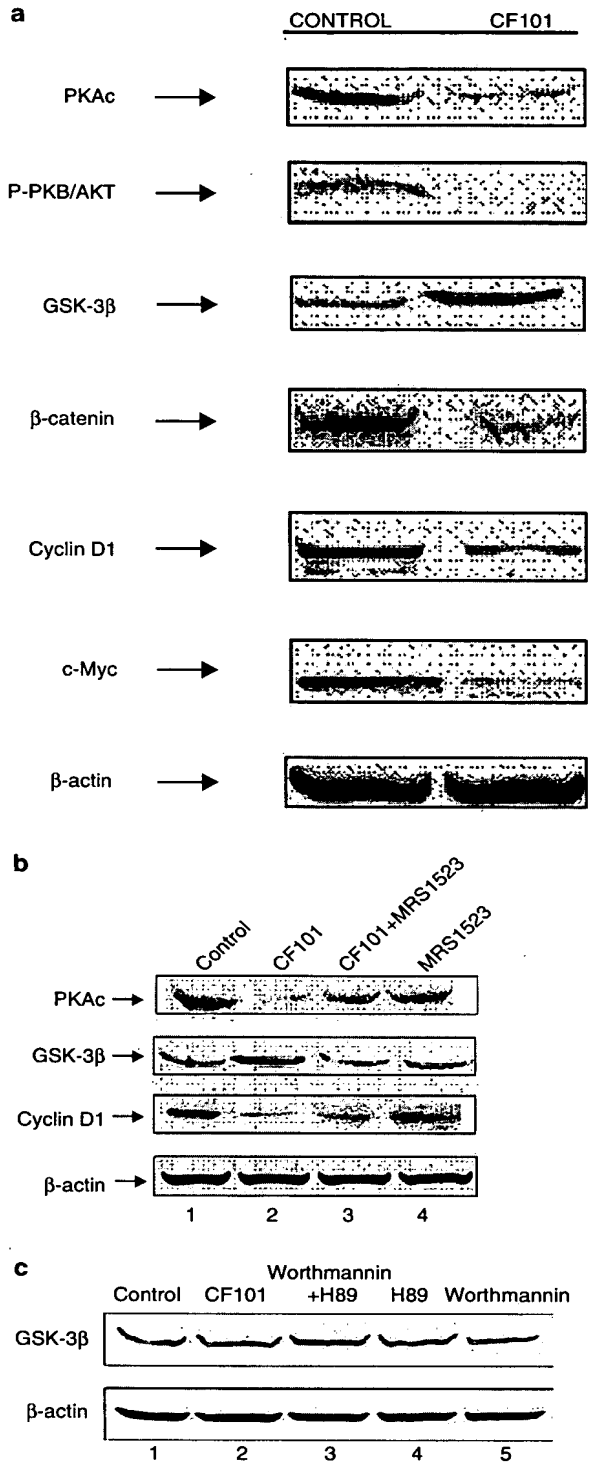



**Figure 1** Inhibition of colon carcinoma cell growth in nude mice and modulation of cell growth-regulatory proteins in tumor lesions. HCT-116 cells were subcutaneously engrafted to nude mice. CF101 (10 $\mu$g/kg) treatment was initiated when tumor reached a size of 150 mm$^3$, and was given twice daily for 21 consecutive days. On day 21, the mice were killed 2 or 16 h after CF101 treatment. Tumor lesions were removed and protein extracts were prepared. (**a**) Tumor size was measured every 4 days. The curve represents a comparison between the vehicle and CF101-treated groups. (**b**) Immunoblots showing the effect of CF101 on cell growth-regulatory proteins derived from the colon carcinoma tumor lesions. $A_3AR$ was downregulated 2 h after treatment and fully expressed after 16 h. Downstream cell growth-regulatory proteins were modulated upon CF101 treatment

PKB in mediating cell response to CF101, their activity was mimicked by H89 and Worthmannin (PKA and PKB/Akt inhibitors, respectively). Figure 2c depicts an increase in GSK-3$\beta$ level upon treatment with the two inhibitors.




### *CF101 deregulates GSK-3β and downstream key signaling proteins*

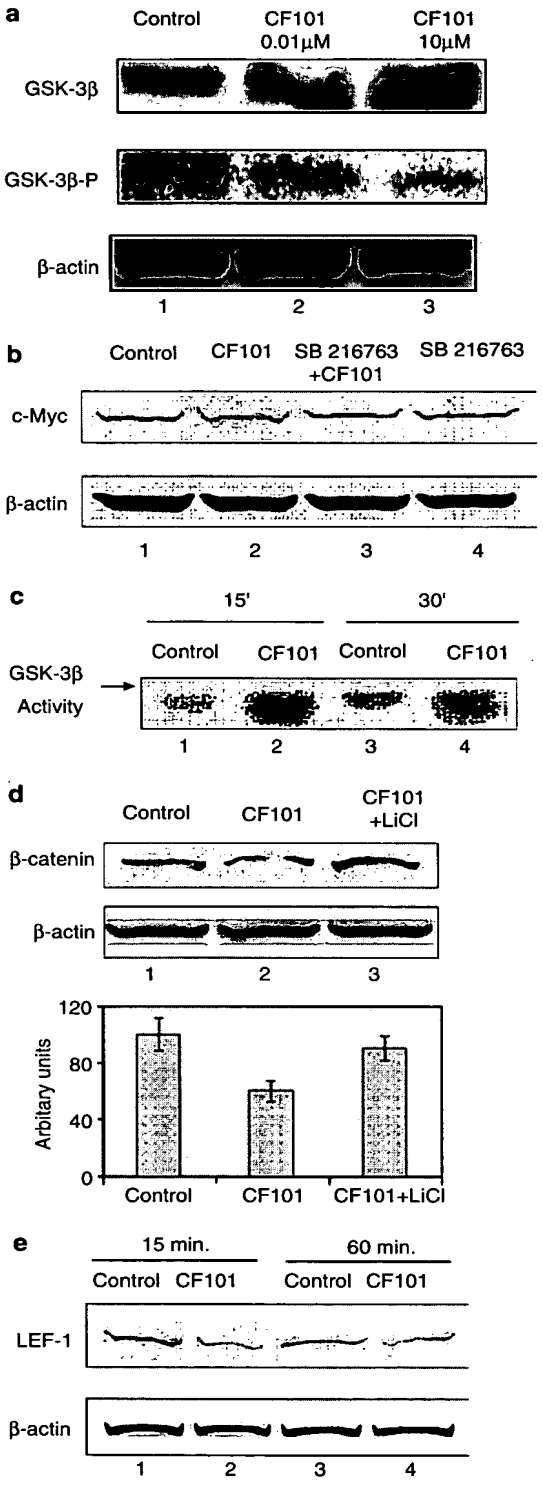
The next set of experiments was carried out to assure that CF101 decreased cyclin D1 and c-Myc levels via modulation of GSK-3$\beta$. We therefore compared the active nonphosphorylated GSK-3$\beta$ level to its nonactive phosphorylated form. Consistent with the former data, we found that, upon CF101 treatment, the nonphosphorylated form was upregulated, whereas the phosphorylated one was decreased (Figure 3a). SB216763, an inhibitor to GSK-3$\beta$, counteracted the ability of CF101 to downregulate c-Myc, confirming that this response was GSK-3$\beta$ mediated (Figure 3b). Furthermore, marked increase in the activity of GSK-3$\beta$ was also noted after 15 and 30 min (Figure 3c). To assess whether the decrease in $\beta$-catenin is mediated via its phosphorylation by GSK-3$\beta$, HCT-116 cells were treated with lithium chloride that inhibits the serine/threonine phosphorylation activity of GSK-3$\beta$. Indeed, lithium treatment reversed the decrease in $\beta$-catenin expression level ($36+3.4\%$, $P<0.002$), confirming that this response is GSK-3$\beta$ mediated (Figure 3d). In addition, the nuclear level of LEF-1 in the CF101-treated cells was downregulated (Figure 3e), supporting the notion that less $\beta$-catenin was associated with LEF-1 and subsequently translocated to the nucleus.

### *Effect of CF101 on the level and transcription activity of NF-κB*

Activated PKB/Akt can phosphorylate IκB kinase, leading to further phosphorylation events and the release of NF-κB from its complex with IκB. Accordingly, we examined whether the downregulation of PKB/Akt will affect the protein expression and DNA-binding capacity of NF-κB, also known to induce cyclin D1 and c-Myc transcription. Indeed, decreased NF-κB level was seen in protein extracts derived from CF101-treated HCT-116 cells (Figure 4a). This decrease was blocked when the antagonist MRS 1523 was present in the culture medium together with CF101, demonstrating the specificity of this response. Moreover, electrophoretic mobility shift assay (EMSA) conducted with cell nuclei extracts revealed marked reduction in NF-κB

**Figure 2** Modulation of cell growth-regulatory proteins in HCT-116 colon carcinoma cells upon CF101 treatment *in vitro*. (a) Immunoblots showing the effect of 10 nM CF101 on the expression levels of PKAc, PKB/Akt, GSK-3$\beta$, $\beta$-catenin, cyclin D1 and c-Myc in HCT-116 cells. Serum-starved cells (for 18 h) were treated for 15 min with CF101 in the presence of 1% FBS. (b) To test the specificity of this response, the antagonist MRS 1523 (100 nM) was introduced to the culture system. Immunoblots showing the effect of CF101 on the cell growth-regulatory proteins in the presence and absence of MRS 1523 are depicted. (c) Immunoblots showing the effect of H89 (10 $\mu$M) and Worthmannin (100 nM) on the expression level of GSK-3$\beta$

DNA-binding capacity at 15, 30 and 60 min, suggesting a reduction in the NF-κB transcription activity at these time points (Figure 4b).




## Discussion

In the present study, we followed the downstream signaling events taking place subsequent to $A_3AR$ activation, resulting in tumor growth inhibition. These studies were conducted in a xenograft nude mice model, and were confirmed *in vitro*.

In mice treated chronically for 20 days with CF101, receptor protein downregulation was noted shortly after CF101 administration. Later on, prominent $A_3AR$ expression was noted, demonstrating that $A_3AR$ was fully expressed in the tumor cells after chronic treatment with CF101. These fluctuations may be attributed to receptor internalization, degradation and re-synthesis, which occurs subsequent to receptor activation. These data are the first to show $A_3AR$ expression *in vivo*, and

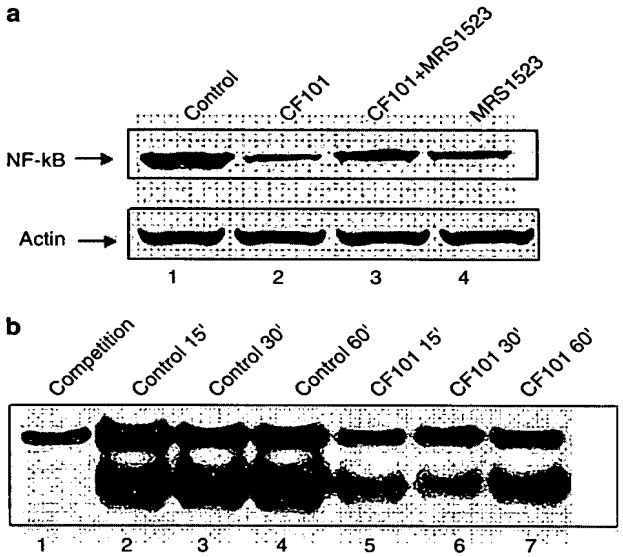



**Figure 4** NF-κB expression level in cell lysates and EMSA in nuclear extracts. HCT-116 colon carcinoma cells were incubated for 15, 30 and 60 min at 37°C with 10 nM CF101. (**a**) WB analysis of whole-cell protein extracts conducted at 15 min of incubation in the absence and presence of the antagonist MRS 1523 (100 nM) and (**b**) EMSA of HCT-116 nuclear extracts at different time points

**Figure 3** Increase in GSK-3β expression level and activity upon treatment of HCT-116 cells with CF101 leads to decreased β-catenin expression level. Cells were depleted from serum for 18 h and treated with vehicle (control) or with CF101 (10 nM or 10 μM) in the presence of 1% FBS for the times and concentrations indicated. (**a**) The expression of nonphosphorylated GSK-3β and phosphorylated GSK-3β (GSK-3β-P) was determined in cell protein extracts by WB analysis. (**b**) The ability of CF101 to inhibit the expression level of c-Myc was counteracted by SB216763, an inhibitor of GSK-3β (**c**). GSK-3β activity in HCT-116 colon carcinoma cells was incubated for 15 and 30 min at 37°C with 10 μM CF101. (**d**) HCT-116 cells were treated with CF101 (10 nM) for 15 and 30 min in the presence and absence of lithium chloride. The latter counteracted the decrease in β-catenin expression level, indicating that the response is GSK-3β mediated. (**e**) LEF-1 analysis in the nuclear extracts of HCT-116 cells treated with CF101, as detailed above, for 30 and 60 min

support the notion that colon carcinoma cells do not develop 'resistance' or 'tolerance' to chronic treatment with a synthetic $A_3AR$ agonist. Supporting the above is our recent publication demonstrating that, upon activation of B16-F10 melanoma cells with IB-MECA, $A_3AR$ was internalized and sorted to the lysosome for degradation. Later on, the receptor was resynthesized and recycled to the cell surface (Madi *et al.*, 2003).

In the present study, receptor functionality was demonstrated by the modulation in the expression level of key signaling cell growth-regulatory proteins downstream to receptor activation. This included downregulation of PKAc and PKB/Akt and upregulation of GSK-3$\beta$. Additionally, the protein expression levels of $\beta$-catenin, LEF-1 and the two oncogenes cyclin D1 and c-Myc were found to be decreased.

These results are in accordance with our previous studies, which showed decreased PKAc and PKB/Akt levels upon treatment of B16-F10 melanoma cells with IB-MECA (Fishman *et al.*, 2002b; Madi *et al.*, 2003). PKAc is the catalytic subunit of PKA, known to be activated subsequent to increase in cAMP level. Activation of $A_3AR$ is known to decrease adenylyl cyclase activity and cAMP formation, resulting in a decline in PKAc level. PKB/Akt has recently been shown to be phosphorylated and thereby activated by PKAc (Fang *et al.*, 2000). The PI3K arm was reported to be upregulated upon $A_3AR$ activation via the $\beta\gamma$-subunit (Schutle and Fredholm, 2002), leading to an increase in the phosphorylated form of PKB/Akt. Here, we show that, in colon carcinoma cells, downregulation of PKB/Akt takes place upon receptor activation, suggesting that in tumor cells modulation of the PKA arm is the dominant event, leading to the downregulation of PKB/Akt. PKAc and PKB/Akt utilize GSK-3$\beta$ as a substrate and, upon phosphorylation, GSK-3$\beta$ activity is inhibited. The latter has been widely implicated in cell homeostasis, by its ability to phosphorylate a broad range of substrates including $\beta$-catenin, a key component of the Wnt pathway (Ferkey and Kimelman, 2000). In normal cells, GSK-3$\beta$ phosphorylates $\beta$-catenin, thereby inducing its ubiquitination and degradation by the proteosome system (Morin, 1999). However, in tumor cells, GSK-3$\beta$ fails to phosphorylate $\beta$-catenin, leading to its accumulation in the cytoplasm. It then translocates to the nucleus, where it acts in concert with LEF-1 to induce the transcription of the cell cycle progression genes such as cyclin D1 and c-Myc (Kolligs *et al.*, 2002).

In previous studies, we showed that $A_3AR$ activation induced downregulation of cyclin D1 and c-Myc in melanoma and prostate carcinoma cells, via deregulation of some Wnt signaling proteins (Fishman *et al.*, 2002b, 2003; Madi *et al.*, 2003). We thus assume that the decreased expression level of $\beta$-catenin is responsible for the diminished level of cyclin D1 and c-Myc.

In the present study, we examined the effect of CF101 on HCT-116 colon carcinoma cells, known to be mutated in the $\beta$-catenin gene (CTNNB1) (Lovig *et al.*, 2002). Mutations of CTNNB1 were found at the GSK-3$\beta$ consensus phosphorylation site of $\beta$-catenin, that is, a deletion of serine 45 that occurs at a putative phosphorylation target of GSK-3$\beta$ (Ilyas *et al.*, 1997). Surprisingly, we found that downregulation of $\beta$-catenin expression, which occurred upon CF101 treatment, was subsequent to an increase in the level of GSK-3$\beta$, notwithstanding the previously described, aforementioned mutation. Moreover, treatment of the cells with lithium, which directly inhibits the activity of GSK-3$\beta$, reversed the $\beta$-catenin level to that of the control. It thus seems that CF101 circumvents the inability of GSK-3$\beta$ to phosphorylate $\beta$-catenin, leading to its susceptibility to degradation. Support for the involvement of $\beta$-catenin in the downregulation of cyclin D1 and c-Myc may be found in the data showing that nuclear level of LEF-1 was downregulated upon CF101 treatment. Furthermore, the GSK-3$\beta$ inhibitor SB216763 counteracted the ability of CF101 to downregulate c-Myc, thus confirming that the events downstream to $\beta$-catenin are also mediated via GSK-3$\beta$.

An additional mechanism which may account for the downregulation of c-Myc and cyclin D1 is the direct phosphorylation of the two oncogenes by GSK-3$\beta$. It was recently shown that GSK-3$\beta$ phosphorylates c-Myc at Thr-58 and cyclin D1 at Thr-286, thereby triggering their degradation (Alt *et al.*, 2000; Sears *et al.*, 2000).

The decreased level of PKB/Akt prompted us to examine the involvement of an additional important signaling protein, NF-$\kappa$B, known to be phosphorylated and activated by PKB/Akt and additional downstream kinases. Since NF-$\kappa$B is also involved in the transcription of cyclin D1 and c-Myc (Karin *et al.*, 2002), its decreased level may also attribute to the diminished expression of the two cell cycle genes.

The Wnt and the NF-$\kappa$B signaling pathways are interconnected at the level of cyclin D1 and c-Myc. Both $\beta$-catenin and NF-$\kappa$B control the transcription of these genes, thereby acting as a sensor for growth signals. Taken together, we propose here a model in which activation of the $A_3AR$ induces modulation of PKAc and PKB, which on one hand upregulates GSK-3$\beta$, leading to phosphorylation and ubiquitination of $\beta$-catenin. On the other hand, remarkably, the similarity between the *in vitro* and *in vivo* data supports the notion that signaling proteins involved with the Wnt and NF-kB pathways are responsible for the observed modulation of cell growth-regulatory proteins.

The finding that cyclin D1 and c-Myc were downregulated upon $A_3AR$ activation both *in vitro* and *in vivo* is highly important in light of the bulk literature showing that most human cancers are characterized by overexpression of the two oncogenes (Hosokawa and Arnold, 1998; Parrella, 2001; Masuda *et al.*, 2002). In some malignancies, overexpression of these proteins may serve as a marker of poor prognosis (Chana *et al.*, 2002; Nguyen *et al.*, 2003). The importance of these two oncogenes in modulating the tumorigenic response was evidenced by the introduction of an antisense cyclin D1 or c-Myc sequence to malignant cells. This led to the inhibition of growth, the induction of apoptosis and the enhancement of sensitivity to chemotherapeutic agents (Van Waardenburg *et al.*, 1997). Additionally, Jain *et al.*

(2002) showed that brief MYC inactivation induced sustained loss of neoplastic phenotype.

Taken together, the molecular model that transpires upon activation of $A_3AR$ with CF101 includes downregulation of PKAc with a subsequent decrease in PKB/Akt expression level. This may lead on one hand to upregulation of the unphosphorylated form of GSK-3$\beta$ and the phosphorylation and ubiquitination of $\beta$-catenin, resulting in the inhibition of translation of cyclin D1 and c-Myc. Additional events taking place downstream to PKB/Akt include decreased expression and DNA-binding capability of NF-$\kappa$B, leading also to downregulation of cyclin D1 expression level.

The capability of CF101, a small orally bioavailable molecule, to downregulate cyclin D1 and c-Myc levels both *in vitro* and *in vivo* suggest that the compound is an attractive candidate to be developed as an anticancer agent.

## Materials and methods

### *Reagents*

CF101 is a GMP grade of the $A_3AR$ agonist 1-deoxy-1-amino]-9*H*-purine-9-yl]-*N*-methyl-(-D-ribofuranuronamide) (IB-MECA), and was synthesized for Can-Fite BioPharma by Albany Molecular Research Inc., Albany, NY, USA. MRS 1523, a highly selective $A_3AR$ antagonist, was purchased from RBI/Sigma (Natick, MA, USA). For both reagents, a stock solution of 10 mM was prepared in DMSO and further dilutions in RPMI medium were performed. Lithium chloride and H89 were purchased from Sigma Israel, and SB216763 was purchased from Biomol Research Laboratories Inc. (Plymouth, USA). RPMI, fetal bovine serum (FBS) and antibiotics for cell cultures were obtained from Beit Haemek, Haifa, Israel.

Rabbit polyclonal antibodies against murine and human A3AR and the cell growth-regulatory proteins PKAc, PKB/Akt, c-Myc, GSK-3$\beta$, phosphor-specific GSK-3$\beta$ (S9), $\beta$-catenin, cyclin D1 and LEF-1 and $\beta$-actin were purchased from Santa Cruz Biotechnology Inc., CA, USA.

### *Effect of CF101 on the growth of HCT-116 colon carcinoma in nude mice and assessment of $A_3AR$ expression and cell growth-regulatory proteins in tumor lesions*

Mice were maintained on a standardized pelleted diet and supplied with tap water. Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Can-Fite BioPharma, Petah Tikva, Israel.

Nude male Balb/*c* mice, aged 2 months, weighing an average of 20 g, were obtained from Harlan Laboratories, Jerusalem, Israel. HCT-116 colon carcinoma cells ($1.2 \times 10^6$) were subcutaneously injected into the flank of the mice. When tumor reached 150 $mm^3$ in size, CF101 (10 $\mu$g/kg body weight) was administered orally twice daily for 20 days. The control group was treated orally twice daily with the vehicle only. Tumor size (width ($W$) and length ($L$)) was measured twice weekly with a caliber, and calculated according to the following formula: tumor size $= (W)^2 \times L/2$.

After 20 days of treatment and prior to terminating the study, the CF101-treated mice were divided into two groups. (A) mice treated for 20 days with CF101 and killed 16 h after last treatment; (B) mice treated for 20 days with CF101, received additional treatment on day 21 and killed 2 h later. Tumor lesions from the two groups and the control were then excised, homogenized (Polytron, Kinematica) and protein was extracted. WB analysis was carried out to determine the $A_3AR$ expression level and additional cell growth-regulatory proteins. Each group contained 15 mice and the study was repeated three times. The results depicted are a representative experiment.

### *WB analysis*

WB analysis of the following samples was carried out: (A) tumor lesions derived from CF101 and vehicle-treated nude mice inoculated with HCT-116 colon carcinoma cells (detailed above). (B) HCT-116 human colon carcinoma cells were serum starved overnight and then incubated with CF101 (10 nM or 10 $\mu$M) in the presence and absence of MRS 1523 (100 nM), H89 (10 $\mu$M), Worthmanin (100 nM), and/or SB216763 (1 $\mu$M) for time periods, as specified below, at 37°C. Samples were rinsed with ice-cold PBS and transferred to ice-cold lysis buffer (TNN buffer, 50 mM Tris buffer pH = 7.5, 150 mM NaCl, 0.5% NP-40). Cell debris were removed by centrifugation for 10 min, at 7500 *g*. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. Equal amounts of the sample (50 $\mu$g) were separated by SDS–PAGE, using 12% polyacrylamide gels. The resolved proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked with 1% BSA and incubated with the desired primary antibody (dilution 1:1000) for 24 h at 4°C. Blots were then washed and incubated with a secondary antibody for 1 h at room temperature. Bands were recorded using BCIP/NBT color development kit (Promega, Madison, WI, USA). Data presented in the different figures are representative of at least four different experiments.

### *Preparation of nuclear extracts*

Nuclear extract proteins from CF101-treated and control HCT-116 cells were prepared by incubating the cells for 15 min on ice in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT and 0.5 mM PMSF. Following incubation, Nonident P-40 (10%) was added, cells were vortexed for 10 s and centrifuged. The pellet was resuspended in a buffer containing 20 mM HEPES (pH = 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF, rocked on a shaker for 15 min at 4°C and centrifuged. Protein was quantified utilizing Bio-Rad protein assay dye reagent.

### *GSK-3β immunoprecipitation*

HCT-116 human colon carcinoma cells were serum starved overnight and then incubated with CF101 (10 $\mu$M) for 30 min at 37°C. After isolating protein, 300 $\mu$g from each sample was removed for immunoprecipitation. The samples were cleared by incubating for 2 h with 1 $\mu$g/sample of rabbit IgG and 10 $\mu$l/sample of GammaBind Sepharose (Pharmacia, Piscataway, NJ, USA). After centrifuging, the supernatants were transferred to a tube containing 3 $\mu$g/sample of Ab against GSK-3$\beta$ bound to GammaBind Sepharose, and then rotated at 4°C overnight. The beads were subsequently washed three times with high salt buffer (1 M Tris-HCl pH 7.4, 0.50 M NaCl, and 1% Nonidet P-40) and three times with lysis buffer without protease inhibitors. The immunoprecipitated complexes were used in a kinase activity assay.

*GSK-3β activity assay*

After immunoprecipitating GSK-3$\beta$ from HCT-116 cells, the protein-containing pellet was washed twice with kinase buffer (20 mM $MgCl_2$, 25 mM HEPES, 20 mM glycerophosphate, 20 mM *p*-nitrophenylphosphate, 20 mM sodium orthovanadate and 2 mM DTT). The pellet was then suspended in 20 $\mu$l kinase buffer and the following ingredients were added: 20 $\mu$M ATP, 5 $\mu$Ci ATP (BLU 002Z; DuPont-NEN, Boston, MA, USA) and 10 $\mu$g myelin basic protein (MBP; Sigma). The total volume of sample plus additions at this point was 25 $\mu$l. The reaction was continued for 30 min at 25°C and then stopped by the addition of 25 $\mu$l/sample of 2 × sample buffer. The samples were boiled for 5 min, then run on a 12% SDS–PAGE gel. The gel was dried, and autoradiography performed to visualize the $^{32}$P-labeled MBP.

*EMSA of NF-κB*

To carry out the gel shift assay, double-stranded oligonucleotides for the consensus sequence of NF-kB (5′-AGTT-GAGGGGACTTTCCCAGGC-3′) were end-labeled with $^{32}$ATP (Amersham) using polynucleotide kinase (Promega). Nuclear protein extracts (3 $\mu$g) were incubated for 30 min at room temperature with the end-labeled DNA (1 $\mu$g) in binding buffer containing 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM DTT, 25 mM EDTA, 20% glycerol, 50 mM Tris-HCl pH 7.5 and 2 $\mu$g/sample of poly (dI-dC), in a final volume of 25 $\mu$l. Competition with unlabeled oligonucleotide of NF-κB binding sequence at a 100-fold molar excess was used to analyse specific bands. The reaction product was analysed by 6% nondenaturating polyacrylamide gel electrophoresis. The specific bands were visualized by X-ray autoradiography.

*Statistical analysis*

The results were evaluated using the Student's *t*-test, with statistical significance set at $P<0.05$. Comparison between the mean values of different experiments was carried out.

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